

REPORT DOCUMENTATION

AD-A280 461

ived
04-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing the collection of information, gathering and maintaining the data needed, and completing and reviewing the collection of information, including suggestions for reducing this burden, to Washington Headquarters Service, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Project, Washington, DC 20503.

existing data sources, by other aspect of this report. 1215 Jefferson 3503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE 1994		3. REPORT TYPE AND DATES COVERED Reprint																					
4. TITLE AND SUBTITLE (see title on reprint)				5. FUNDING NUMBERS PE: NWED QAXM WU: 00132																					
6. AUTHOR(S) Patchen et al.																									
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Armed Forces Radiobiology Research Institute 8901 Wisconsin Ave. Bethesda, MD 20889-5603				8. PERFORMING ORGANIZATION REPORT NUMBER SR94-4																					
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20814-4799				10. SPONSORING/MONITORING AGENCY REPORT NUMBER																					
11. SUPPLEMENTARY NOTES																									
12a. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution unlimited.				12b. DISTRIBUTION CODE																					
13. ABSTRACT (Maximum 200 words)																									
<table border="1"> <tr> <td colspan="2">Accession For</td> </tr> <tr> <td>NTIS CRA&I</td> <td><input checked="" type="checkbox"/></td> </tr> <tr> <td>DTIC TAB</td> <td><input type="checkbox"/></td> </tr> <tr> <td>Unannounced</td> <td><input type="checkbox"/></td> </tr> <tr> <td colspan="2">Justification</td> </tr> <tr> <td colspan="2">By</td> </tr> <tr> <td colspan="2">Distribution /</td> </tr> <tr> <td colspan="2">Availability Codes</td> </tr> <tr> <td>Dist</td> <td>Avail and/or Special</td> </tr> <tr> <td>A-1</td> <td>20</td> </tr> </table>						Accession For		NTIS CRA&I	<input checked="" type="checkbox"/>	DTIC TAB	<input type="checkbox"/>	Unannounced	<input type="checkbox"/>	Justification		By		Distribution /		Availability Codes		Dist	Avail and/or Special	A-1	20
Accession For																									
NTIS CRA&I	<input checked="" type="checkbox"/>																								
DTIC TAB	<input type="checkbox"/>																								
Unannounced	<input type="checkbox"/>																								
Justification																									
By																									
Distribution /																									
Availability Codes																									
Dist	Avail and/or Special																								
A-1	20																								
14. SUBJECT TERMS				15. NUMBER OF PAGES 12																					
				16. PRICE CODE																					
17. SECURITY CLASSIFICATION OF REPORT UNCLASSIFIED		18. SECURITY CLASSIFICATION OF THIS PAGE UNCLASSIFIED		19. SECURITY CLASSIFICATION OF ABSTRACT UNCLASSIFIED																					
				20. LIMITATION OF ABSTRACT																					

DTIC
ELECTE
S **D**
JUN 20 1994
G

DTIC QUALITY INSPECTED 2

7

Single and Combination Cytokine Therapies for Treatment of Radiation-induced Hematopoietic Injury: Effects of *c-kit* Ligand and Interleukin-3

M.L. PATCHEN¹, R. FISCHER¹, T.J. MACVITTIE¹,
F. SEILER² AND D.E. WILLIAMS³

¹Department of Experimental Hematology, Armed Forces Radiobiology Research Institute, 8901 Wisconsin Avenue, Bethesda, MD 20889-5603, USA, ²Research Laboratories of Behringwerke AG, Marburg, Germany, and ³Immunex Corporation, Seattle, WA, USA

94-18886



Abstract

Following radiation exposures, severe hematopoietic depression can result from injury to hematopoietic stem and progenitor cell populations. In recent years, a variety of recombinant cytokines have been demonstrated to possess hematopoietic activity. While some cytokines are lineage restricted in their activity, others such as *c-kit* ligand and interleukin-3 (IL-3) appear to be capable of affecting early multilineage hematopoietic cell populations. Using a B₆D₂F₁ murine model of severe ⁶⁰Co radiation-induced hematopoietic hypoplasia, we have evaluated the ability of *c-kit* ligand (recombinant murine mast cell growth factor, rmMGF) and rmIL-3 to accelerate hematopoietic regeneration when administered either alone or in combination following radiation exposure. Hematopoietic regeneration was based on spleen and bone marrow spleen colony forming unit (CFU-s₁₂) and granulocyte-macrophage progenitor cell (GM-CFC) recoveries. MGF alone, administered subcutaneously (s.c) on days 1-17 postirradiation at 100 µg/kg/day or 200 µg/kg/day, accelerated bone marrow and splenic GM-CFC as well as splenic CFU-s recoveries in a direct dose-dependent manner. IL-3 alone (100 µg/kg/day, s.c. on days 1-17)

94 6 12 056

84 *Preclinical and clinical update on growth factors*

also accelerated splenic GM-CFC and CFU-s recoveries. When these cytokines were co-administered (100 µg/kg/day each, s.c. on days 1-17), GM-CFC and CFU-s recoveries greater than those produced by either cytokine alone were observed. These studies illustrate a potential role for combined MGF and IL-3 in the treatment of radiation-induced hematopoietic injury.

Introduction

One of the most recent cytokines implicated in hematopoietic regulation is *c-kit* ligand, also known as mast cell growth factor (MGF), steel factor (SLF), and stem cell factor (SCF) (1-3). The *c-kit* ligand has been ascribed numerous hematopoietic and nonhematopoietic effects, although it was initially identified and purified based on its ability to stimulate mast cell growth (2-5). Multiple studies have focused on the *in vitro* effects of this factor, demonstrating that alone it has limited hematopoietic activity, but when combined with other hematopoietic cytokines, it synergizes to increase both the number and size of colonies generated from hematopoietic progenitors (3-11), and in some instances, to increase the replating potential of primitive progenitors (12). Furthermore, in combination with such factors, *c-kit* ligand also synergistically enhances the *in vitro* expansion of hematopoietic progenitors grown in liquid cultures (13-15). These effects are thought to result not only from the ability of *c-kit* ligand to potentiate progenitor cell proliferation but also from its ability to enhance progenitor cell survival (14,16).

IL-3, also known as multi-CSF, has previously been shown to enhance hematopoietic regeneration in irradiated animals based on recovery of peripheral blood white cells and platelets (17). Because *in vitro* studies have demonstrated synergistic hematopoietic stimulation produced by *c-kit* ligand combined with IL-3, we evaluated whether co-administration of these cytokines *in vivo* would synergize to further accelerate hematopoietic regeneration following radiation-induced hematopoietic hypoplasia.

Materials and methods

CYTOKINES

Recombinant murine *c-kit* ligand (MGF), was provided by Immunex (Seattle, WA). Recombinant murine IL-3 was provided by Behringwerke AG (Marburg, Germany). Cytokines were expressed in yeast and purified to homogeneity as previously described (10,18). Endotoxin contamination of cytokines was below the limit of detection using the Limulus amoebocyte lysate assay. MGF was administered subcutaneously (s.c.) in a 0.1 ml volume at the dose of 100 or 200 µg/kg; IL-3 was administered s.c. at the dose of 100 µg/kg. In combination studies, mice received each cytokine at a separate injection site. All injections

were initiated 1 day following irradiation and continued daily for 17 days. Control mice were injected with an equal volume of sterile saline.

MICE

B₆D₂F₁ female mice (~20 g) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were maintained in an AAALAC (American Association for Accreditation of Laboratory Animal Care) accredited facility in Micro-Isolator cages on hardwood-chip contact bedding and were provided commercial rodent chow and acidified water (Ph 2.5) *ad libitum*. Animal rooms were equipped with full-spectrum light from 6 a.m. to 6 p.m. and were maintained at 70°F +/- 2°F with 50% +/- 10% relative humidity using at least 10 air changes per hour of 100% conditioned fresh air. Upon arrival, all mice were tested for *Pseudomonas* and quarantined until test results were obtained. Only healthy mice were released for experimentation. All animal experiments were approved by the Institute Animal Care and Use Committee prior to performance.

IRRADIATION

The ⁶⁰Co source at the Armed Forces Radiobiology Research Institute was used to administer bilateral total-body gamma radiation. Mice were placed in ventilated Plexiglas containers and irradiated with 7.75 Gy at a dose rate of 0.4 Gy/min. Dosimetry was performed using ionization chambers (19) with calibration factors traceable to the National Institute of Standards and Technology. The tissue-to-air ratio was determined to be 0.96. Dose variation within the exposure field was < 3%.

CELL SUSPENSIONS

Cell suspensions for each assay represented tissues from three normal, irradiated, or irradiated and cytokine-treated mice at each time point. Cells were flushed from femurs with 3 ml of McCoy's 5A medium (Flow Labs, McLean, VA) containing 10% heat-inactivated fetal bovine serum (Hyclone Labs, Logan, UT). Spleens were pressed through a stainless steel mesh screen, and the cells were washed from the screen with 6 ml medium. The number of nucleated cells in the suspensions was determined by Coulter counter. Femurs and spleens were removed from mice euthanized by cervical dislocation.

SPLEEN COLONY FORMING UNIT ASSAY

Exogenous spleen colony forming units (CFU-s) were evaluated by the method of Till and McCulloch (20). Recipient mice were exposed to 9 Gy of total-body radiation to reduce endogenous hematopoietic stem cells. Bone marrow or

spleen cells were intravenously (i.v.) injected into the irradiated recipients 3-5 h later. Twelve days after transplantation, the recipients were euthanized by cervical dislocation, and their spleens were removed. The spleens were fixed in Bouin's solution, and grossly visible spleen colonies were counted. Each treatment group consisted of five mice.

GRANULOCYTE-MACROPHAGE COLONY-FORMING CELL ASSAY

Hematopoietic progenitor cells committed to granulocyte and/or macrophage development were assayed using a double-layer agar granulocyte-macrophage colony forming cell (GM-CFC) assay in which mouse endotoxin serum (5% v/v) was added to feeder layers as a source of colony stimulating factors (21). Colonies (> 50 cells) were counted after 10 days of incubation in a 37°C humidified environment containing 5% CO₂. Triplicate plates were cultured for each cell suspension.

STATISTICS

Results of replicate experiments were pooled and are represented as the mean \pm standard error (SE) of pooled data. Statistical differences were determined by Behrens-Fisher t-test analysis. Significance level was set at $p < 0.05$.

Results

The ability to accelerate hematopoietic regeneration in a murine model of severe radiation-induced hematopoietic hypoplasia was used to evaluate the potential of MGF and IL-3 to induce hematopoietic progenitor cell expansion *in vivo*. In preliminary studies, it was determined that a sublethal 7.75 Gy ⁶⁰Co radiation exposure induced severe hematopoietic hypoplasia from which recovery (especially in the spleen) became evident between days 14 and 17 post-irradiation (Figure 7.1). Based on these preliminary studies, subsequent studies evaluating the ability of cytokines to accelerate hematopoietic recovery focused on evaluation of bone marrow and splenic CFU-s and GM-CFC recoveries on days 14 and 17 post-irradiation.

The effects of MGF on CFU-s and GM-CFC recoveries in sublethally irradiated mice are illustrated in Figure 7.2 and Figure 7.3, respectively. At either the 100 µg/kg/day or 200 µg/kg/day dose, MGF alone accelerated bone marrow and splenic GM-CFC recovery. The 200 µg/kg/day MGF dose also accelerated splenic CFU-s recovery; however, no effect on bone marrow CFU-s recovery was observed at either MGF dose. Following administration of IL-3 alone, both splenic CFU-s (Figure 7.4B) and GM-CFC (Figure 7.5B) numbers were increased compared to saline-treated mice by day 17 postirradiation. No

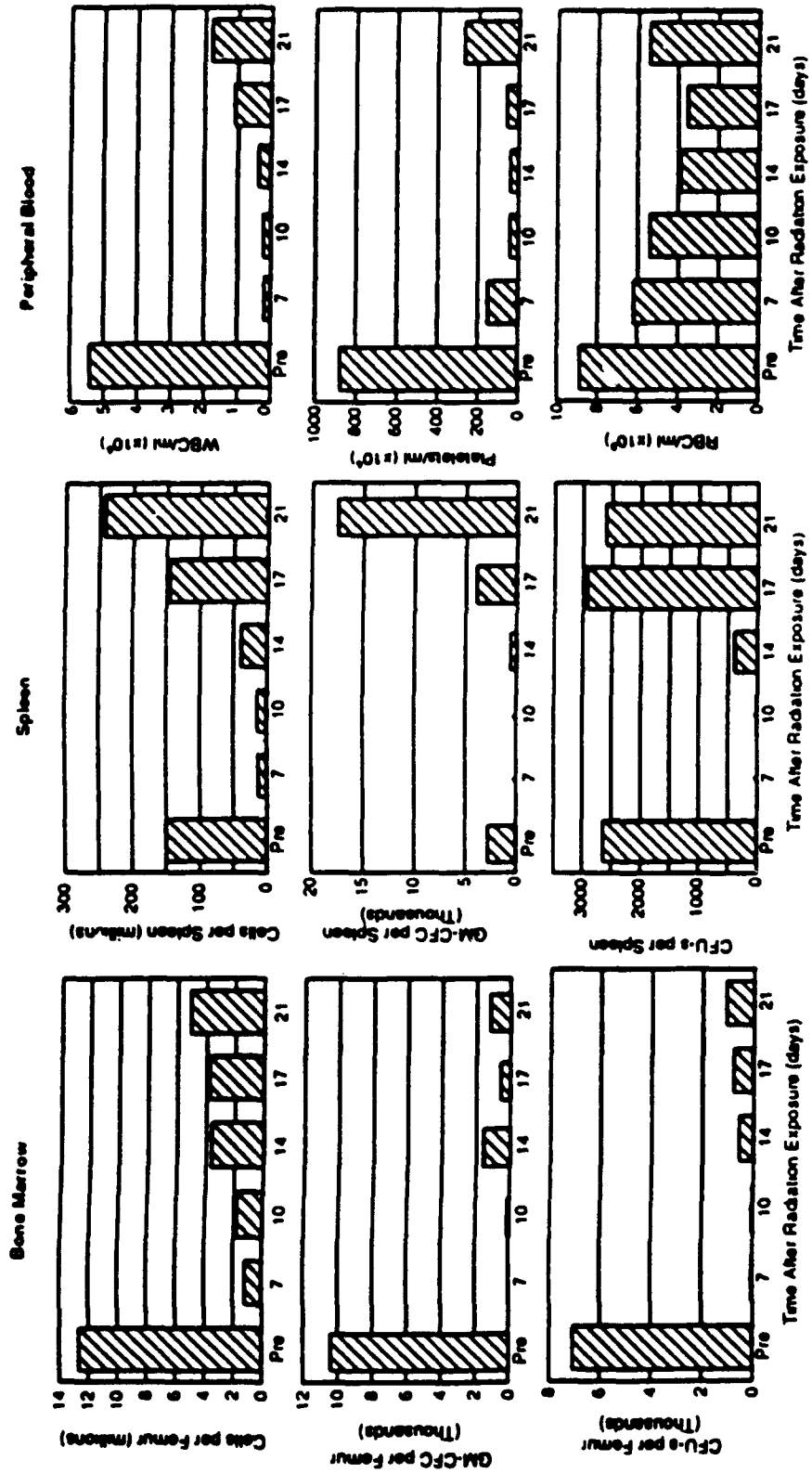
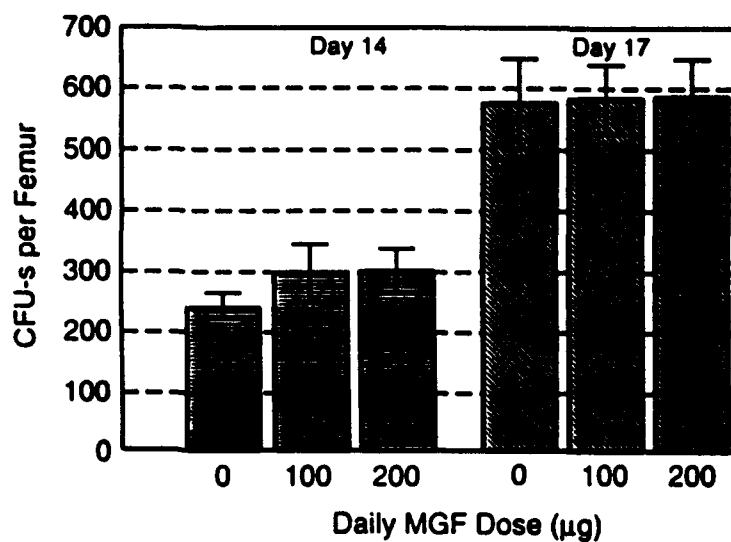
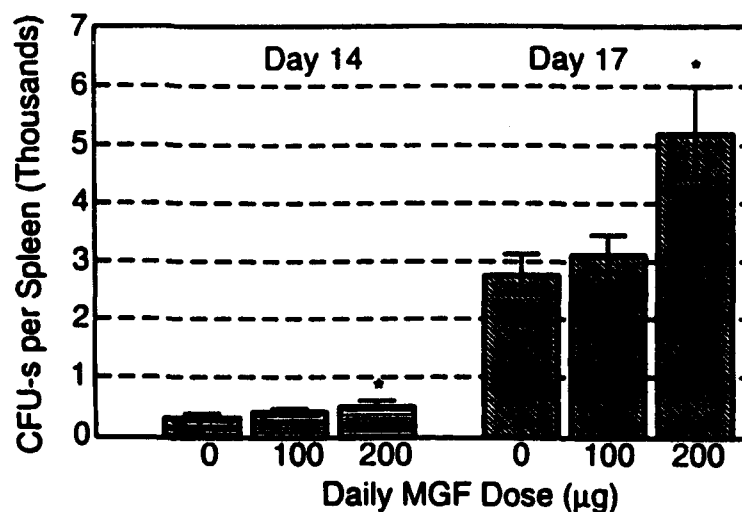


Figure 7.1 Hematopoietic suppression and recovery following a sublethal 7.75 Gy ⁶⁰Co radiation exposure in B₆D₂F₁ mice.



Normal Control $7,223 \pm 150$



Normal Control $2,722 \pm 100$

Figure 7.2 Effects of MGF administration (100 or 200 µg/kg/day, x17 day, s.c.) on bone marrow (A) and splenic (B) CFU-s recovery on days 14 and 17 after a 7.75 Gy radiation exposure in $B_6D_2F_1$ mice. Mean \pm SE; * $p < 0.05$, with respect to saline controls.

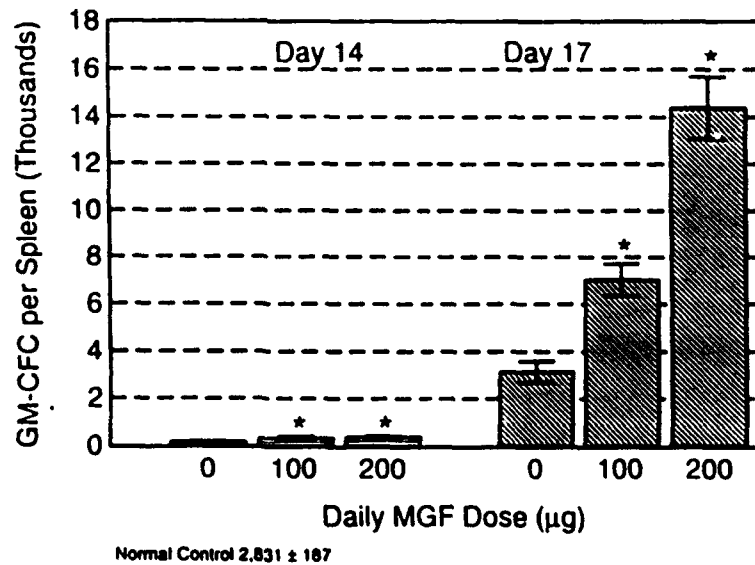
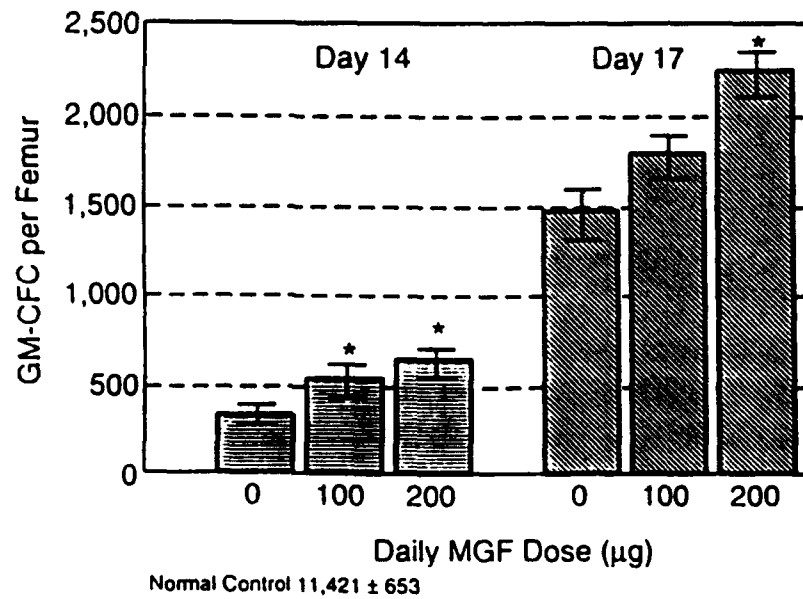


Figure 7.3 Effects of MGF administration (100 or 200 µg/kg/day, x17 day, s.c.) on bone marrow (A) and splenic (B) GM-CFC recovery on days 14 and 17 after a 7.75 Gy radiation exposure in B₆D₂F₁ mice. Mean ± SE; * p < 0.05, with respect to saline controls.

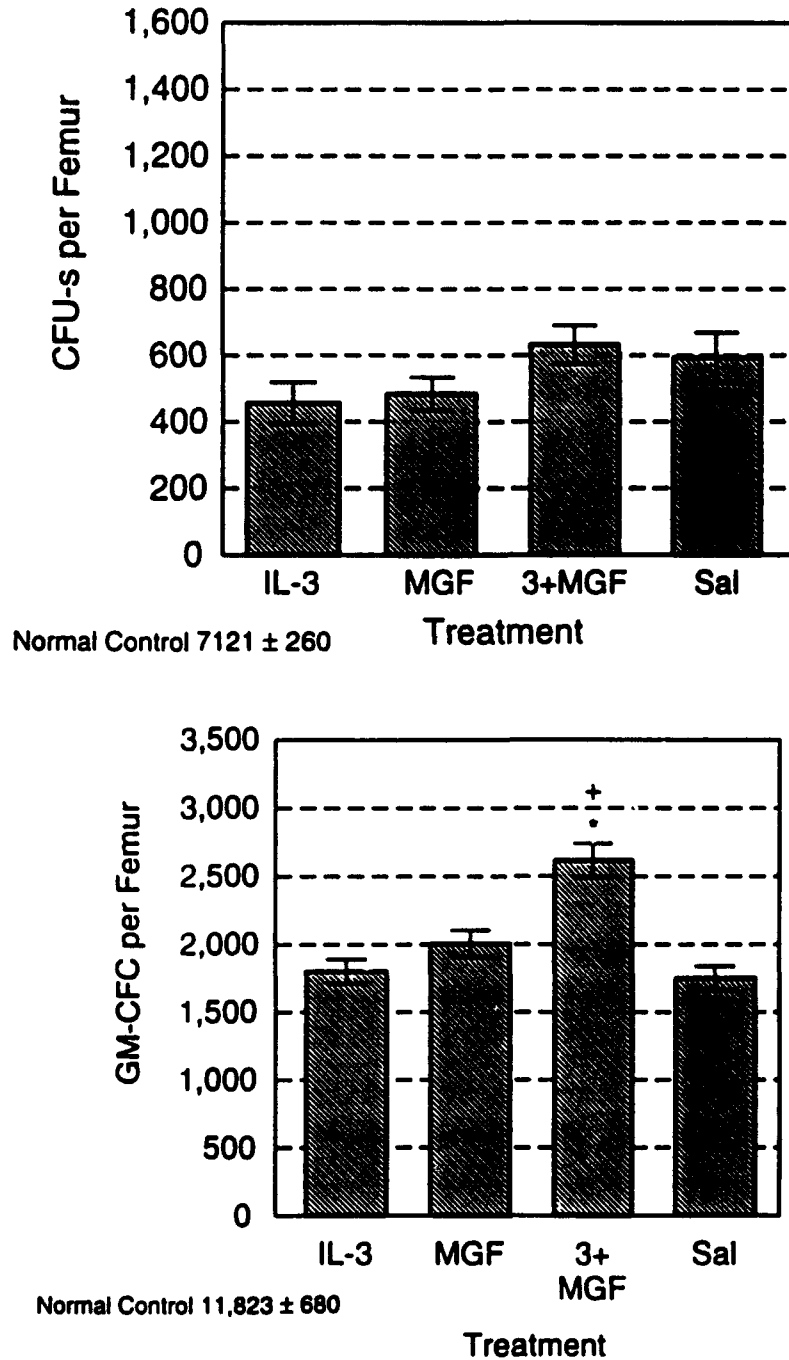


Figure 7.4 Effects of IL-3, MGF, and MGF plus IL-3 (each 100 µg/kg/day, x17 day, s.c.) on bone marrow (A) and splenic (B) CFU-s recovery on day 17 after a 7.75 Gy radiation exposure in B₆D₂F₁ mice. Mean ± SE; * p < 0.05, with respect to saline controls; + p < 0.05, with respect to IL-3 values.

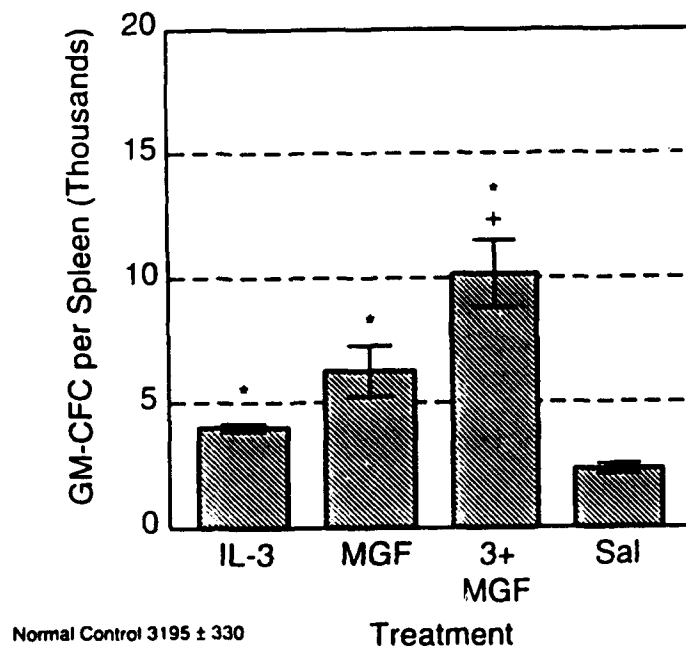
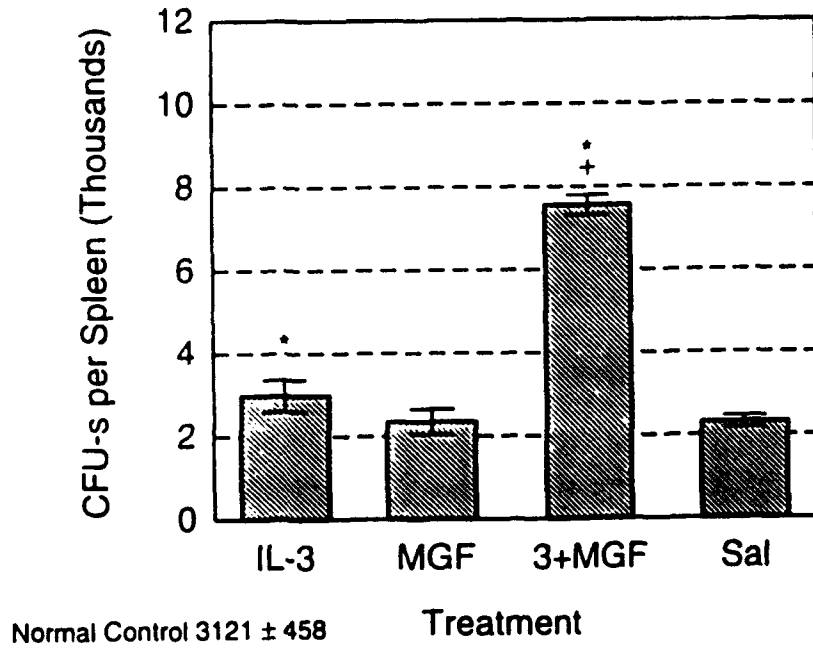


Figure 7.5 Effects of IL-3, MGF, and MGF plus IL-3 (each 100 $\mu\text{g/kg/day}$, x17 day, s.c.) on bone marrow (A) and splenic (B) GM-CFC recovery on day 17 after a 7.75 Gy radiation exposure in $B_6D_2F_1$ mice. Mean \pm SE; * $p < 0.05$, with respect to saline controls; + $p < 0.05$, with respect to MGF values.

IL-3 effects, however, were observed on bone marrow recovery. When MGF was administered to sublethally irradiated mice in combination with IL-3, CFU-s (Figure 7.4) and GM-CFC (Figure 7.5) recoveries greater than those induced by MGF alone or IL-3 alone were observed, with splenic effects being more dramatic than bone marrow effects.

Discussion

Sustained hematopoietic recovery following chemotherapy or radiation exposure requires surviving pluripotent stem cells to self-renew as well as to differentiate into multipotent and committed progenitors capable of giving rise to functional mature cells. In recent years, administration of single hematopoietic growth factors, including G-CSF, GM-CSF, MGF, and IL-6, has been shown to stimulate hematopoietic regeneration following radiation- or chemotherapy-induced myelosuppression. In addition, some cytokine combinations, such as GM-CSF plus IL-3, have proven to surpass the effectiveness of single agents. Because *c-kit* ligand, *in vitro*, has been shown to synergize with IL-3 in stimulating progenitor cell proliferation and expansion, we hypothesized that administration of MGF in combination with this cytokine *in vivo* may further improve hematopoietic regeneration beyond that obtained with only MGF or only IL-3.

Our studies demonstrate that in irradiated mice:

1. MGF alone can accelerate hematopoietic regeneration.
2. IL-3 alone can also accelerate hematopoietic regeneration, and
3. when MGF and IL-3 are co-administered, hematopoietic recovery greater than that produced by either single cytokine can be obtained.

It remains to be determined whether the apparent lack of effect of MGF, IL-3, or the combination of these cytokines on bone marrow CFU-s regeneration concomitant with significant splenic CFU-s regeneration may be due to cytokine mediated bone marrow CFU-s mobilization. In spite of this, these studies suggest a potential role for combined MGF plus IL-3 in the treatment of hematopoietic hypoplasia.

References

1. Witte ON. *Steel* locus defines new multipotent growth factor. *Cell* 63:5, 1990
2. Williams DE, Eisenman J, Baird A, Rauch C, Van Ness K, March CJ, Park LS, Martin U, Mochizuki DY, Boswell HS, Burgess GS, Cosman D, Lyman SD. Identification of a ligand for the *c-kit* proto-oncogene. *Cell* 63:167, 1990
3. Zsebo KM, Wypych J, McNeice IK, Lu HS, Smith KA, Karkare SB, Sachdev RK, Yuschenkoff VN, Brikett NC, Williams LR, Satyagal VN, Tung W, Bosselman RA,

- Mendiaz EA, Langley KE. Identification, purification, and biological characterization of hematopoietic stem cell factor from buffalo rat liver-conditioned medium. *Cell* 63:195, 1990
4. Nocka K, Buck J, Levi E, Besmer P. Candidate ligand for the c-kit transmembrane kinase receptor: KL, a fibroblast derived growth factor stimulates mast cells and erythroid progenitors. *EMBO J* 9:3287, 1990
5. Williams DE, DeVries P, Namen AE, Widmer MB, Lyman SD. The Steel factor. *Develop Biol* 151:368, 1992
6. Martin FH, Suggs SV, Langley KE, Lu HS, Ting J, Okino KH, Morris F, McNeice IK, Jacobsen FW, Mendiaz EA, Birkett NC, Smith KA, Johnson MJ, Parker VP, Flores JC, Patel AC, Fischer EF, Erjavec HO, Herrera CJ, Wypych J, Sachdev RK, Pope JA, Leslie I, Wen D, Lin CH, Cupples RL, Zsebo KM. Primary structure and functional expression of rat and human stem cell factor DNAs. *Cell* 63:203, 1990
7. McNeice IK, Langley KE, Zsebo KM. Recombinant human stem cell factor synergizes with GM-CSF, G-CSF, IL-3, and Epo to stimulate human progenitor cells of the myeloid and erythroid lineages. *Exp Hematol* 19:226, 1991
8. Broxmeyer HA, Hangoc G, Cooper S, Anderson D, Cosman D, Lyman SD, Williams DE. Influence of murine mast cell growth factor (c-kit ligand) on colony formation by mouse marrow hematopoietic progenitor cells. *Exp Hematol* 19:143, 1991
9. Williams N, Bertoncello I, Kavnoudias H, Zsebo KM, McNeice I. Recombinant rat stem cell factor stimulates the amplification and differentiation of fractionated mouse stem cell populations. *Blood* 79:58, 1992
10. Anderson DM, Lyman SD, Baird A, Wingnall JM, Eisenman J, Rauch C, March CJ, Boswell S, Gimpel SD, Cosman D, Williams DE. Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms. *Cell* 63:235, 1990
11. deVries P, Brasel KA, Eisenman JR, Alpert AR, Williams DE. The effect of recombinant mast cell growth factor on purified murine hematopoietic stem cells. *J Exp Med* 173:1205, 1991
12. Carow CE, Hangoc G, Cooper SH, Williams DE, Broxmeyer HE. Mast cell growth factor (c-kit ligand) supports the growth of human multipotential progenitor cells with a high replating potential. *Blood* 78:2216, 1991
13. Moore MAS. Clinical implications of positive and negative hematopoietic stem cell regulators. *Blood* 78:1, 1991
14. Bernstein ID, Andrews RG, Zsebo KM. Recombinant human stem cell factor enhances the formation of colonies by CD34+ and CD34+Lin- cells, and the generation of colony-forming cell progeny from CD34+Lin- cells cultured with interleukin-3, granulocyte colony-stimulating factor, or granulocyte-macrophage colony-stimulating factor. *Blood* 77:2316, 1991
15. Brandt J, Briddell RA, Srour EF, Leemhuis TB, Hoffman R. The role of c-kit ligand in the expansion of human hematopoietic progenitor cells. *Blood* 79:634, 1992
16. Heyworth CM, Whetton AD, Nicholls S, Zsebo K, Dexter TM. Stem cell factor directly stimulates the development of enriched granulocyte-macrophage colony-forming cells and promotes the effects of other colony-stimulating factors. *Blood* 80:2230, 1992
17. MacVittie TJ, Monroy RL, Farese AM, Patchen ML, Seiler FR, Williams DE. Cytokine therapy in canine and primate models of radiation-induced marrow aplasia. *Behring Inst Mitt* 90:1, 1991
18. Urdal DL, Mochizuki D, Conlon PJ, March CJ, Remerowski ML, Eisenmann J, Ramthun C, Gillis S. Lymphokine purification by reversed phase high performance liquid chromatography. *J Chromatogr* 296:171, 1984

94 Preclinical and clinical update on growth factors

19. Schulz J, Almond PR, Cunningham JR, Holt JG, Loevinger R, Suntharalingam N, Wright KA, Nath R, Lempert D. A protocol for the determination of absorbed dose for high energy photon and electron beams. *Med Phys* 10:741, 1983
20. Till JE, McCulloch EA. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res* 14:213, 1961
21. Patchen ML, MacVittie TJ. Hematopoietic effects of intravenous soluble glucan administration. *J Immunopharmacol* 8:407, 1986